Functional Analyses of Mitochondrial Proteins in *Saccharomyces cerevisiae*

PhD Thesis

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Abbreviations

ATP	adenosine triphosphate
CIT1, CIT2	mitochondrial and peroxisomal citrate synthase respectively
CoA	coenzyme A
cyaY, CyaY	the gene and the protein of the *E. coli* frataxin homologue
DNA	dezoxyribonucleic acid
E. coli	*Escherichia coli*
FRDA	Friedreich ataxia
GalP	galactose promoter
hGP	human Graves protein
IPMS	α-isopropylmalate synthase
α-IPM	α-isopropylmalate
β-IPM	β-isopropylmalate
LEU4, LEU5	gene encoding IPMS and its hypothetical analogue
MDH	malate dehydrogenase
MPP	mitochondrial is processing peptidase
mtDNA	mitochondrial DNA
PCR	polymerase chain reaction
PMS	post mitochondrial supernatant
S. cerevisiae	*Saccharomyces cerevisiae*
SDH	succinate dehydrogenase
Tim22p	trans inner membrane translocase
YFH1, Yfh1p	the gene and the protein of the yeast frataxin homologue

Inroduction

It is a well established technique in the field of molecular biology to use different organisms to study the function of protein homologs. There are two mayor advantages to use a simple organism as a model. One of them, when the originally found phenotype couldn't be studied in that organism because that is so complicated, it is not possible to use genetic engineering, or on the other hand when phenotype found in a simple organism could be more easily explained in a more complex background. One of the most frequently used organism as a model, is the baker's yeast, *Saccharomyces cerevisiae*, often mentioned as the "work horse" of molecular biology. This simple organism has all the advantages what the scientists may whish: fast replication and easy genetic manipulation. However it is also suitable to study more complicated processes, since as an eukaryote this organism already carries subcellular compartments like nucleus and mitochondria.

The thesis based on the functional analyses of the homolog protein pairs, the human Graves disease protein (hGP) and its homolog the yeast Leu5p, furthermore the bacterial and yeast frataxin homologs the CyaY and the Yfh1p.

The Leu5p and the hGP

There are several vital processes taking place in the mitochondria, like oxidative phosphorylation, tricarboxylic acid cycle, β-oxidation, partially the urea cycle, the biosynthesis of heme and a few amino acids. There are a large number of substances necessary to be transported, specifically and fast enough between the matrix of the mitochondria and the cytosol, for the satisfying metabolic activity of mitochondria, since the inner membrane is not permeable for these components. There are a relatively

large number of transport proteins serving for these processes, of which there is a special group called mitochondrial carriers. The members of this group are sharing about 20-40 % of amino acid identity. There are 35 carriers known in yeast, however only 13 substrates of these proteins identified to date. The *YHR002w* gene of the *S. cerevisiae* encodes such a protein (P38702) having all the characteristic elements of mitochondrial carriers. This gene has been subcloned earlier to identify a second α-izopropylmalate dehydrogenase, in addition to the already well characterized Leu4p. The gene named that time as *LEU5*. Since the single Δleu4 is not, but Δleu4 Δleu5 double mutant is leucine auxotroph, it was suggested the encoded a Leu4p analog. The sequence analyses of *LEU5* reviled that, Leu5p could be integrated in the mitochondrial inner membrane, and may be not even involved in leucine synthesis. The Leu5p has a human homolog called hGP a protein involved in Graves disease, with unknown function. The amino acid composition of these two proteins are 35 % identical.

The frataxin, Yfh1p and CyaY

Friedreich's ataxia (FRDA) is the most common inherited ataxia transmitted in an autosomal fashion, with an occurrence of 1 or 2 in 50.000 individuals. Characteristic symptoms include ataxia of the limbs, areflexia, muscle weakness and skeletal deformities, cardiomyopathy, sensory loss, and increased incidence of diabetes mellitus. FRDA is caused by severely reduced levels of frataxin, a protein localized to the mitochondrial matrix. This 210 amino acid residues protein has been thought to be involved in iron homeostasis since its discovery. However the function of frataxin is still controversial, its importance is reflected by the conservation during the evolution.

It has been showed, that frataxin is able to bind Fe^{2+} in vitro, and as an iron chaperon also able to donate it for the biosynthesis of ISC and heme, and to repair the ISC of aconitase. It seems to be the mayor role of frataxin is to detoxify the excess iron of the mitochondria and to chaperon bioavailable iron for important processes.

The yeast frataxin homolog (Yfh1p) is one of the most studied form of the frataxin homologs. Deletion of the encoding gene, *YFH1* (Δyfh1) causes a growth defect on non fermentable carbon source, leads to a tendency for loosing mitochondrial DNA (mtDNA), cells became sensitive against oxidative stress and iron accumulates in the mitochondria. However the lack its bacterial homolog, the *E. coli* CyaY (*ΔcyaY*) causing no obvious effect to the cells. In vitro this protein also behaves similarly to its homologs. Since CyaY can not be studied *in vivo* in its original background since deletion of *cyaY* doesn't result change in phenotype, most likely due to its redundancy in this environment, we expressed this gene in a frataxin depleted *S. cerevisiae*.

Perspectives

1. - identify the function of Leu5p
   - complement a Δleu5 yeast strain with the human Leu5p homolog hGP
2. - delete the *cyaY* in *E. coli* (*ΔcyaY*) and characterize the strain
   - complement a Yfh1p depleted yeast strain with CyaY

Methods

Bacterial and yeast strains, producing mutant strains, medias

To subclone and to produce DNA plasmids, DH5α, to produce recombinant proteins, BL21, XLBlue and DH5α *E. coli* strains were used. *CyaY* deletion was carried out in a CLT42 strain, using a Zeocine antibiotic resistance gene. W303-1A or W303-1B yeast strains were used as a wild type or the following mutant yeast strains were used: Δleu5; Δcit2; Δleu5Δcit2; Δcor1; Δcox6; Δflx1; Δmir1 and GK178. Gene deletion in yeast, were made by one step gene disruption. For *E. coli* Luria or minimal liquid media were used, the cultures were propagated at 37°C with shaking. Same media were used to prepare plates, containing 2% agar. In both liquid or solid media contained 0.4 % of different carbon sources.

For yeast YP rich, or synthetic media (S) were used, supplemented with glucose, galactose, glycerin or ethanol carbonsources, in liquid or solid form. All yeast strains were grown at 30°C. In the strain GK178 the expression of *YFH1* is controlled by a very tight controlled mutant *GAL1* promoter (*gal1**), were the *YFH1* was repressed glucose.

Expression and purification of recombinant proteins

The encoding gene of hGP were amplified by PCR from cDNA, isolated from Jurkat celline, and cubcloned into pYes yeast expression vector. For the production of

His-tagged recombinant CyaY, the encoding gene was subcloned into pQE9 *E. coli* expression vector, using a PCR amplified fragment from wild type *E. coli* chromosomal DNA. The protein was purified by Ni-agarose beads. Wild type CyaY was purified by chromatography in a similar way to that of Yfh1p, due to the similarity of the two proteins. For the expression of *CyaY* in yeast the p426GPD yeast expression vector was used. Since the frataxin is localized to mitochondria in eukaryotes, to target CyaY to the mitochondria the leader peptide of Yfh1p was fused to the N terminal of CyaY. The same vector including a wild type *YFH1*, and an empty vector served as a positive and a negative control respectively. Yeast MPP was expressed and purified by a method published earlier. The ferrochelatase protein used in ferrochelatase assay was a kind gift of Dr. Gloria C. Ferreira (University of Florida, Tampa, FL, USA).

Enzymatic activity assays, determination of the concentration of metabolites

The samples used for the assays were prepared by disrupting the cells wit glass beads, using Yeast Buster, or collecting different fractions at mitochondria isolation. The activity of IPMS was assayed by a fluorimetric method. The activities of malate dehydrogenase (MDH), aconitase, and succinate dehydrogenase (SDH) were assayed by spectrophotometric methods. Concentration of CoA was measured from the deproteinated samples by a flourimetric, citrate levels were determined enzymatically. The levels of cytochromes were estimated from isolated mitochondria by spectrophotometric method.

Results

The function of Leu5p and hGP

Leu5p located in the inner membrane of the mitochondria

In order to show Leu5p localized to the inner membrane of the mitochondria we studied the import and the exact localization of the protein. [35S] radioactive labeled Leu5p protein were incubated in the presence of isolated, energized mitochondria, then the mixture were treated with proteinase K. The Leu5p showed partial resistance against the enzyme, what means the protein was taken up by the mitochondria. Furthermore the most of the photolytic resistance lost when the outer membrane was broken up by hypotonic shock, showing that the Leu5p exposed to the inner membrane space. The imported protein was resistant to extraction by treatment with alkaline buffers, indicating integration into the membrane. Import of Leu5p into a Tim22p-depleted mitochondria was strongly reduced compared to wild-type organelles, and thus appears to follow the carrier-specific protein import pathway.

Phenotypic consequences of the deletion of LEU5

To initiate the functional investigation of Leu5p, the entire coding region of *LEU5* was deleted (Δleu5). In comparison to wild-type cells, mutant cells lacking LEU5 showed similar growth on rich media containing glucose but displayed strongly retarded growth on rich media containing glycerol. However this strain didn't show auxotrophy for leucine. No significant differences in the cytochrome spectra or in oxidative phosphorylation of Δleu5 mitochondria were observed compared to wild-type organelles. From these data we reasoned that, the Leu5p, as a member of the carrier

family, mediates the transport of a substrate required for proper function of an intra mitochondrial biosynthetic process.

Leu5p is required for accumulation of CoA inside mitochondria

To identify the substrate of Leu5p, we took advantage of the phenotypical observations made for the inactivation of the *LEU4* and *LEU5* genes. The combined, but not the single, mutation of the two genes was reported to result in an auxotrophy for leucine. In Δleu4 cells the α-IPM, necessary for the following step in leucine synthesis is exclusively produced in the mitochondria by Leu9p localized to the mitochondrial matrix. From this piece of data we reasoned that Leu5p, as a mitochondrial carrier, may transport a compound necessary for the reaction in which α-IPM formed. In our experiments it was identified that among the potential candidates, CoA is the substrate what could be transported by Leu5p. This finding has been proven by the strongly reduced CoA content of Δleu5 strain compared to wild type.

The relationship of Leu5p and Cit2p

To show an *in vivo* evidence for the Δleu5 cells reduced mitochondrial CoA content, this genotype was combined with the deletion of the peroxisomal citrate synthase (Δleu5Δcit2). This strain behaved similarly to Δcit1Δcit2, showed *pet* phenotype, was unable to use glycerol as a carbon source, and showed auxotrophy for glutamate, from which we reasoned the citrate synthase located in the mitochondria (cit1p) was unable to perform at a satisfying level due to strongly reduced CoA level.

The hGP functionally complements the defect of Δleu5

The significant homology between hGP and yeast Leu5p suggests a similar function of the two proteins. To test this idea, Δleu5 cells were transformed with a plasmid carrying the hGP gene. The resulting Δleu5/hGP cells were able for utilizing nonfermentable carbon sources and the activity of IMPS, the citrate and the CoA levels of mitochondria restored close to that of the wild type. Our data demonstrate that the human protein can at least partially replace Leu5p function and suggest a role of hGP in the accumulation of CoA in mitochondria.

Study of CyaY

Phenotypic consequences of the deletion of cayY

The *cyaY* gene of *E. coli* was deleted by the homologues recombination of the Zeocine antibiotic resistance gene (*zeo*) to the chromosome. The resulting strain (*ΔCyaY*) was investigated in several conditions where other species, like *S. cerevisiae* are showing dramatic phenotypic changes. However *ΔCyaY* didn't show any obvious phenotype, including auxotrophy for any nutrient, defect in growth rate, sensitivity against oxidative stress or alteration in activity of iron-sulfur cluster enzymes. Together, these observations suggest that the function of CyaY may be functionally redundant in *E. coli*, due to the possible presence of one or more suppressors. May be the biological function of CyaY only necessary in such a special condition, what not even reproducible artificially in laboratory. We therefore reasoned that yeast cells depleted of the endogenous Yfh1p would be suitable to interrogate the function of CyaY in iron delivery and iron detoxification in a non redundant setting.

E. coli CyaY partially complements frataxin depleted yeast

Since frataxin in eukaryotes is localized to mitochondria, we engineered a construct coding for a chimeric protein (Yfh1–CyaY) consisting of the mitochondrial matrix targeting signal of Yfh1p fused in frame with the N-terminus of CyaY.

This construct was subcloned into a constitutively expressing yeast expression vector (p426GDP), and the resulting vector was used to transform the *S. cerevisiae* GK178 strain. In this strain, endogenous expression of *YFH1* is regulated by a tightly controlled mutant *GAL1* promoter (*gal1**), that provides a close to *yfh1Δ* state in the presence of glucose. Growing the cells in the presence of glucose only Yfh1-CyaY hybrid protein was detectable, but not Yfh1p. *In vivo* processing of Yfh1–CyaY generated two products. One of them was also detectable after in vitro processing, thus we reasoned that the Yfh1–CyaY hybrid protein imported and processed by mitochondria.

CyaY complements frataxin depleted yeast

Next, we investigated whether Yfh1–CyaY was able to suppress the phenotypic changes associated with Yfh1p depletion. After frataxin depletion the strain containing CyaY was able to utilize nonfermentable carbon source comparable to the wild type, whereas the non complemented strain were growing very poorly. CyaY was able to protect cells similarly to the Yfh1p, when the cells were exposed to oxidative stress generated by MMS or H_2O_2. When the maintenance of respiratory function was analyzed during treatment with iron, we found that CyaY could make the cells a bit less tolerant against this effect than the Yfh1p did. The activity of iron-sulfur cluster containing enzymes, like aconitase and SDH, in the presence of Yfh1-CyaY was restored to about 80-85% of wild type level.

CyaY restores heme synthesis

Lastly we investigated whether Yfh1–CyaY was also able to restore heme synthesis in frataxin depleted yeast strain. Whereas the cytochromes were under the detection limit in the Yfh1p depleted strain, the presence of Yfh1–CyaY was associated with cytochrome content close to that of wild type strain. Based on this observation we suspected that CyaY was able to donate iron to ferrochelatase for the biosynthesis of heme. This hypothesis was evidenced in an *in vitro* reaction using recombinant proteins. CyaY was able to provide a definitely higher bioavailable Fe^{2+} level than the buffer itself , but was not as efficient as Yfh1p. In this reaction 75:1 iron to protein ratio has been found to be optimal for heme synthesis.

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List of Publications

Publications Served for the Thesis

1. Prohl C, Pelzer W, Diekert K, Kmita H, **Bedekovics T**, Kispal G, Lill R.
   The yeast mitochondrial carrier Leu5p and its human homologue Graves' disease protein are required for accumulation of coenzyme A in the matrix. *Mol Cell Biol.* 2001;21:1089-1097 (IF: 9.8)
2. **Bedekovics T**, Gajdos G, Kispal G and Isaya G.
   Partial conservation of functions between eukaryotic frataxin and the *Esche-richia coli* frataxin homolog CyaY. *FEMS Yeast Res.* 2007; In Press (IF: 2.27)

Other Publications

1. Melegh B, Seress L, **Bedekovics T**, Kispal G, Sumegi B, Trombitas K, Mehes K. Muscle carnitine acetyltransferase and carnitine deficiency in a case of mitochondrial encephalomyopathy. *J Inherit Metab Dis.* 1999;22:827-838 (IF: 1.7)
2. Pal E, **Bedekovics T**, Gati I. Familial scapuloperoneal myopathy and mitochondrial DNA defect. *Eur Neurol.* 1999;42:211-216 (IF: 1.1)
3. Kispal G, Sipos K, Lange H, Fekete Z, **Bedekovics T**, Janaky T, Bassler J, Aguilar Netz DJ, Balk J, Rotte C, Lill R. Biogenesis of cytosolic ribosomes requires the essential iron-sulphur protein Rli1p and mitochondria. *Embo J.* 2005;24:589-598 (IF: 10.4)
4. Kellermayer R, Szigeti R, Keeling KM, **Bedekovics T**, Bedwell DM. Aminoglycosides as potential pharmacogenetic agents in the treatment of Hailey-Hailey disease. *J Invest Dermatol.* 2006;126:229-231 (IF: 4.54)

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Oral and Poster Presentations

Oral Presentation:

1. **Bedekovics T**, Mustaev A and Turnbough CL
   Tethering the nascent transcript to the *Escherichia coli* RNA polymerase active center during reiterative transcription
   10th Biennial Meeting on Post-Initiation Activities of RNA Polymerase, Mountain Lake, VA, USA, **2000**.

Posters:

1. **Bedekovics T**, Gajdos G, Gáti I, Sümegi B and Czopf J
   Detection of mitochondrial DNA deletions in different types of mitochondrial myopathies via polymerase chain reaction
   2nd International Conference of the Hungarian Biochemical Society, Szeged, **1995**.
2. **Bedekovics T** and. Turnbough CL
   The Escherichia coli *pyG* Expression is Negatively regulated by intracellular CTP
   35. Membrántranszport Konferencia, Sümeg, **2005**.
3. **Bedekovics T**, Gajdos G, Kispal G, IsayaG.
   Partial conservation of functions between eukaryotic frataxin and the Escherichia coli frataxin homolog CyaY.
   3rd International Friedreich's Ataxia Scientific Conference, Bethesda, MD, USA, **2006**.